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A PHYSICO-CHEMICAL CHARACTERIZATION OF A NATURAL AGGLUTININ FROM THE MUCUS OF A SLUG *LAEVICAULIS ALTE* (FERUSSAC, 1822)

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ABSTRACT

Natural hemagglutinin, which is highly specific for the glycocalyx of rabbit erythrocytes is identified in the gastropodanmollusc Laevicaulisalte. Of the tissues/fluids of the said species tested for hemagglutination activity, maximum hemagglutin ability is observed in the mucus. Physico–chemical characterization of the mucus agglutinin of L. alte shows optimum agglutinability at pH 7.5 and temperature 55° C. Agglutinability is altered by the inclusion of diverse concentrations of cations or chelators in the buffer. The agglutinability is lost fully, when the mucus is pre-adsorbed with any one erythrocyte that is recognized by the agglutinin. The hemagglutinability of the agglutinin with rabbit erythrocytes is inhibited by the glycoproteins fetuin, α acid glycoprotein, bovine submaxillary mucin, lactoferrin, apotransferrin and the sugars maltose, N-acetyl neuraminic acid, lactose and galactose. Decline in hemagglutination activity when mixed with the asialo rabbit erythrocytes and reduction in hemagglutination inhibition titer when treated with desialylated fetuin, α acid glycoprotein, bovine submaxillary mucin, and lactoferrin reveals the affinity of L. alte mucus agglutinin to sialic acid.

KEYWORDS: Lectin, Mucus Agglutinin, Erythrocytes, Hemagglutination Assay & Hemagglutination Inhibition Assay

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Original Article

INTRODUCTION

Inventions of newlectins have endowed greater insight into the multiplicity and intricacy of lectin repertoires in invertebrates. Lectins are multivalent carbohydrate-binding proteins with the ability to agglutinate erythrocytes, bacteria and other normal and malignant cells displaying more than one saccharide of sufficient complementarity (Barondes, 1981). C- typelectins from molluscs have attracted much attention for their great diversity in structure and activity, and they also provide a model system to understand the molecular basis of how proteins recognize carbohydrates. Because of the wide variety of sugar specificities, C-type lectins are implicated as the indispensable candidates in carbohydrate recognition, capable of discriminating various correlative microbes and in developing biochemical tools.

Lectins react with sugars in glycolipids, glycoproteins, or oligosaccharides and agglutinate erythrocytes via cell surface glycoproteins and glycolipids (Stromberg *et al*., 1991; Sharon, 2008). Their specificity is usually defined in terms of monosaccharide(s) or simple oligosaccharides that inhibit lectin-induced agglutination (Sharon

and Lis, 1972; Goldstein *et al*., 1980). An agglutinin may recognize a part of a sugar (Shimizu *et al*., 1977), a whole sugar (Bretting and Kabat, 1976), their glycosidic linkages (Koch *et al*., 1982; Shibuya *et al*., 1987; Knibbs *et al*., 1991) or a sequence of sugars (Kobiler and Mirelman, 1980; Mauchamp, 1982).

Among protostomian invertebrates that are incapable of synthesizing sialic acids, a sialic acid-specific agglutinin (LFA) was first reported in the slug *Limaxflavus* (Miller *et al*., 1982). Specificity of LFA towards sialic acid led to the development of an assay that facilitated the detection of minute sialic acid changes on the surface of glomerular visceral epithelial cells *in vitro* (Kasinath and Singh, 1992). LFA specificity enabled it to serve as a simple probe in histochemical studies for mapping and labeling, for determining the amount of specific siaic acid residues on the surfaces of cells and tissues and microbial cell walls (Wanchoo *et al*., 2009). LFA also behaved as an antagonists of tetanus toxin and botulinium neurotoxin (Bakry *et al*., 1991). Sialic acids play important roles as ligands in various cells (Rutishauser, 1998). The type of sialic acids and the glycosidic linkages with adjacent sugar in an oligosaccharide differ among pathogenic bacteria (Ravindranath and Cooper, 1984) and human cancer (Higashi *et al*., 1985; Kawai *et al*., 1991). Therefore, lectins specifically recognizing various sialic acids and their carbohydrate binding patterns can be used as bio-agents for identifying various sialyl epitopes on pathogens or in biopsy of malignant tumors. A search for such sialic acid specific lectin is made in a pulmonate slug *L. alte* of the family Veronicellidae of the class Gastropoda. To develop strategies for affinity purification we have studied the physico-chemical properties of the agglutinin.

MATERIAL AND METHODS

Experimental Animals

The slug *L. alte* found in the swampy areas of Holy Cross College campus, and Ramji Rose Nursery, Nagercoil, Plantain garden of Muttaicaud and Rajakkamangalam of Kanyakumari district, Tamil Nadu are handpicked, brought to the Holy Cross College laboratory and reared in plastic troughs by providing the artificially created natural environment using garden soil and decomposing vegetation. The moisture content is maintained by gently sprinkling clean freshwater. They are fed with raw cucumbers.

Collection of Hemolymph

Hemolymph is collected from fresh, healthy, uninjured animals after placing them in a deep freezer (-20°C) for 2 minutes. The hemolymph is withdrawn by inserting a sterile 1.0 ml syringe and 22 gauge needles at the foot from the anterior surface. The hemolymph is poured into pre-chilled centrifuge tubes placed on ice and stored at -20°C.

Preparation of Tissue Extract and Collection of Mucus

The tissue extract is prepared by homogenizing 100 mg of each tissue (oesophagus, stomach, intestine, hepatopancreas and hermaphrodite gland) in 1 ml of cold saline. The homogenized extract is centrifuged at 4000 X g for 10 minutes at 4°C and the supernatant is tested for hemagglutination (HA) activity.

Mucus is collected from the slug *L. alte* by exposing the animal to chloroform vapor for 2 minutes. The mucus released by the animal is collected and thoroughly mixed with cold 0.7% saline at a rate of 100 mg/ml. The mucus that dissolved in the saline is isolated by low speed centrifugation at 4000 x g for 10 minutes at 4^{0}C and the supernatant is tested for HA activity.

Erythrocyte Collection

Blood from different mammals is collected by venipuncture of the ear (rabbit), fore arm (Human A, B, O and horse), cardiac puncture (mouse and rat) and from the slaughter house (ox and pig) directly in modified Alsevier's medium (pH 6.1) containing sodium citrate (30 mM), sodium chloride (77 mM), glucose (114 mM), neomycin sulphate (100 µg/ml) and chloramphenicol (330 µg/ml) at a ratio of 2:8. Erythrocytes are suspended and washed three times by centrifugation at 4,000 x g for 5 minutes with ten volumes of Tris-Buffered Saline (TBS) pH 7.5 (TrisHCl: 50 mM, NaCl: 100 mM, $CaC1_2$: 10 mM) and resuspended in the same as 1.5% suspension.

Hemagglutination Assay

The HA activity of the mucus agglutinin is assayed by measuring its ability to agglutinate erythrocytes. HA assays are performed at 30°C by serial dilution of the mucus (25 µl) with TBS (25 µl) and mixing with 25 µl of 1.5% erythrocyte suspension. HA titer is determined by the visual estimation of erythrocyte agglutination on microtiter plates 1 hour after adding the cells. The HA titer (the units of agglutinin activity) is the reciprocal of the highest dilution of the sample that gave agglutination. To develop strategies for affinity purification, HA assay is also performed with high agglutinating rabbit erythrocytes at different pH, temperature and using buffer with different concentrations of cations calcium, magnesium and manganese and chelator, EDTA

To study the effect of pH on HA titer, the mucus sample is mixed with TBS at specific pH (5-9) at 1:1 ratio and incubated for 1 hour and serially diluted in microtiter plates having TBS of same pH before adding erythrocyte suspension. To study the effect of temperature on HA titer, the mucus sample is incubated for 1 hour at specific temperature (10-70°C) and used for HA assay. To study the effect of cations and chelators on HA titer, the mucus sample incubated for 1 hour in equal volume of TBS containing specific concentration (0.01, 0.1, 1.0 and 10 mM) of cations (calcium, magnesium and manganese) and chelator (EDTA) is used for HA assay.

Cross-Adsorption Test

Packed erythrocytes (rabbit / ox / pig / dog) are prepared by repeated washing of erythrocytes in 0.9% saline by centrifugation at 4,000 X g for 5 minutes until we get a clear pellet. Mucus is mixed with equal volume of packed rabbit / ox / pig / dog erythrocytes and incubated for 18 hours at 4°C with occasional shaking. After centrifugation, the supernatant is analyzed for HA.

Hemagglutination Inhibition (HAI) Assay

Mucus (25 µl) diluted to sub agglutination concentration (dilution at which mucus is able to provide 2 wells HA) is added to each well containing 25 µl of known concentration of serially diluted inhibitors (glycoproteins, mono and oligosaccharides). After incubation for 1 hour, 25 µl of 1.5% rabbit erythrocyte suspension is added. The HAI titer is reported as the reciprocal of the highest dilution of inhibitors giving complete inhibition of agglutination after 1 hour.

Preparation of Asialo Erythrocytes and Desialylated Glycoproteins

A reaction mixture containing 10% washed rabbit erythrocytes in TBS-BSA (pH 6.5) and 140 mU sialidase of *C. perfringens* (Sigma-Type X) is incubated at 37 ° C for 4 hours. Sialidase treated cells are washed with TBS-BSA three times pelleted by low speed centrifugation and used for HA assay.

Desialylated glycoproteins are prepared by incubating 2 mg of glycoprotein with 0.1 unit of *C. perfringens* sialidase (Type X) in 400 µl of 5 mM acetate buffer pH 5.5 for 3 hours at 37^{o}C and used for HAI assay.

RESULTS

Distribution of Hemagglutinins

Of the various tissues of the slug *L. alte* tested for hemaggulutinins, maximum hemaggulutinability was observed in the mucus with rabbit erythrocytes (Table - 1). Hence, further characterizations were restricted to the mucus.

Table 1: Hemaggultinins in the Slug *L. Alte*

Erythrocytes Tested (n=20)	HA Titer		
	Hemolymph	Mucus	Oesophagus
Human A	0	2	4
Human B	0	4	4
Human O	0	4	0
Rat	-	2	32
Rabbit	16	512	8
Pig	8	32	64
Cow	0	4	8
Goat	0	4	-
Dog	2-4	16-32	-
Ox	4	32	8
Horse	-	8	-

Stomach, intestine, hepatopancreas and hermaphrodite gland did not show any agglutination with any of the tested erythrocytes at 100 mg/ml concentration.

HA Activity of the Mucus Agglutinin Following Cross Adsorption

When the mucus of the slug *L. alte* adsorbed to any one of the erythrocytes agglutinated by the mucus agglutinin was used for HA, the agglutinability of the agglutinin to agglutinate the same and other erythrocytes was completely lost (Table – 2)

Table 2: Cross Adsorption Assay of the Mucus Agglutinin of the Slug *L. Alte*

Erythrocytes Adsorbed (n=5)	HA Titer			
	Rabbit	Ox	Pig	Dog
None	512	32	32	16-32
Rabbit	0	0	0	0
Ox	0	0	0	0
Pig	0	0	0	0
Dog	0	0	0	0

Effect of pH, Temperature Cations and EDTA on the HA Titer of the Mucus Agglutinin of the Slug *L. Alte*

The HA titer of the mucus of the slug *L. alte* was sensitive to change in pH (Table - 3) and temperature, showing highest agglutinability at pH 7.5 and temperature 55^{o}C.

Table 3: Effect of Temperature and pH on HA Titer of the Mucus Agglutinin of the Slug *L. Alte*

S. No. (n = 5)	Temperature (°C)	HA Titer	pH	HA Titer
1	15	32	4.0	128
2	20	32	4.5	128-256
3	25	32	5.0	128-256
4	30	32	5.5	128-256
5	35	32	6.0	128-256
6	40	32	6.5	128-256
7	45	32	7.0	128-256
8	50	64-128	7.5	512
9	55	512	8.0	128-256
10	60	64-128	8.5	128
11	65	8	9.0	128
12	70	2	-	-

With the addition of increasing concentrations of calcium, an increase in HA was observed up to 10 mM concentration (HA titer = 512). Addition of magnesium showed a two old reduction in HA titer (HA titer = 64) at all concentrations. Addition of 1 mM manganese increased HA (HA titer = 128 - 256), but with the addition of increasing concentration, agglutinability began to decline and became almost negligible (HA titer = 4) when mixed with 100 mM manganese (Table – 4)

Table 4: Effect of Cations and EDTA on HA Titer of the Mucus Agglutinin of the Slug *L. Alte*

Concentration (mM) (n = 5)	HA Titer			
	Ca	Mg	Mn	EDTA
0	128	128	128	128
0.01	128	64	128	16
0.1	256	64	128	4
1.0	512	64	128	2
10.0	512	64	4	-

Addition of 1.0 mM disodium EDTA showed 32 fold reduction in HA titer of the mucus of the slug *L. alte*, which got further reduced with the addition of increasing concentrations of di sodium EDTA (Table – 4).

Hemagglutination Inhibition Assay of the Mucus Agglutinin of the Slug *L. Alte*

Agglutinablity of the mucus was inhibited by sugars: maltose (HAI titer = 16), N- acetyl neuraminic acid and lactose (HAI titer = 8), galactose (HAI titer = 4), and glycoproteins: fetuin and α acid glycoprotein (HAI titer = 1024), bovine submaxillarymucin (HAI titer = 512), porcine thyroglobulin (HAI titer = 256), lactoferrin (HAI titer = 128), and apotransferrin (HAI titer = 64) (Table – 5).

Table 5: Hemagglutination Inhibition Assay of the Mucus Agglutinin of the Slug *L. Alte*

Inhibitors (n=5)	HAI titer	Min. Conc. required for Inhibition	Relative inhibitiory potency (%)
Sugars (mM)			
Glucose	0	>100	0
Lactose	8	12.5-2.5	25-50
Xylose	0	>100	0
Galactose	4	25	25

Table 5: Contd.,			
Maltose	16	6.25	100
Mannose	0	>100	0
Fructose	0	>100	0
Glucose 6 PO_4	0	>100	0
GalNAc	0	>100	0
ManNAc	0	>100	0
NeuAc	8	>1.25	50
Glycoproteins (µg/ml)			
Fetuin	1024	9.765	100
α acid glycoprotein	1024	9.765	100
Bovine submaxillarymucin	512	19.531	50
P. thyroglobulin	256	39.062	25
Lactoferrin	128	78.125	12.5
Apotransferrin	64	156.25	6.25
PSM	0	>5000	0
Bovine thyroglobulin	0	>5000	0

Effect of Sialidase Treatment of Rabbit Erythrocyte and Glycoproteins on HA and HAI Titer of the Mucus Agglutinin of the Slug *L. Alte*

When asialo rabbit erythrocytes were used for HA assay, 256 fold decrease in HA titer was observed, and HAI titer withdesialylatedfetuin, α acid glycoprotein and lactoferrin (Table – 6).

Table 6: Effect of Neuraminidase Treatment on the Hemagglutination Inhibition Assay of the Mucus Agglutinin of the Slug *L. Alte*

Sialoglycoproteins used (n=5)	HAI Titer with rabbit erythrocytes	
	Untreated	Neuraminidase Treated
Fetuin	1024	16
α-acid glycoprotein	1024	16
Bovine submaxillarymucin	512	8
Porcine thyroglobulin	256	4
Lactoferrin	128	0

DISCUSSIONS

Among various tissues tested, the mucus of the slug *L. alte* recognized rabbit erythrocytes with great specificity. The ability of the slug agglutinin to agglutinate rabbit erythrocytes argues for the specific recognition of the sugars constituting the glycocalyx of these erythrocytes, which serve as receptors to ligands as in the eukaryotic cells (Hakomori, 1973). It has been found that different animal species have characteristic receptor determinants on their erythrocyte surface (Yamakawa and Suzuki, 1953) and interspecies variations (Yasue *et al*., 1978). In *Limaxflavus*, immunostaining of tissues have shown the presence of LFA primarily on the epidermal surface and mucus glands (Kurachi et al., 1998).

Like agglutinins from many other species (Miller *et al*., 1972), the *L. alte* agglutinin is also sensitive to temperature and pH. The biological activity of the mucus agglutinin increased with increase in temperature up to 55^{o}C. However, further increase in temperature and longtime exposure of the agglutinin at 55^{o}C caused a reduction in HA which became almost negligible by 70^{o}C. It is known that the thermal denaturation of proteins is irreversible. Denaturation could be due to the destabilization of sporadic weak interactions of tertiary structure responsible for native conformation of lectin

(Singh and Saxena, 2013). This type of denaturation is attributed to aggregation, autolysis and chemical alteration of residues (Klibanov and Ahern, 1987), which lock the protein in a final state that is unable to fold back to the native structure. Any pH change is associated with a change in the ionization state of the molecule, which in turn, determines the binding forces between enzyme and substrate (Adolph and Lorenz, 1982). More acidic and more alkaline medium are less favorable conditions for hemaggultination activity (Singh and Saxena, 2013).

There was a marked difference in the strength with which lectins bind divalent cations. The binding activities of all lectins were dependent on their metal ion content and when the cations were removed, the lectins lost their carbohydrate binding activity (Lonnerdal *et al*., 1983). Since divalent cations are known to be important in stabilizing the structure of hemagglutinins (Acton and Weinheiner, 1974; Anderson and Good,1975; Finstad *et al*., 1972) the effect of divalent cations on the hemagglutinating activity is examined. It is found that the metallic salt Ca^{2+} significantly enhanced the hemagglutinating activity of the mucus agglutinin while the presence of Mg^{2+} and Mn^{2+} had no significant influence. EDTA, a metal chelator, abolished agglutinability completely, suggesting that Ca^{2+} is essential for hemagglutination of the mucus agglutinin of slug *L. alte* which is released completely from the lectin after treatment with EDTA. These divalent cations could be concerned with holding lectin subunits together or involved in the actual binding site of the molecule (Rogers and Blunden, 1980). The calcium ion helps to bind the protein and carbohydrate by interacting with the OH groups found on the carbohydrate. Calcium also can form a linkage between the carbohydrate and glutamates in the lectin. Binding is further strengthened through hydrogen bonds that form between the lectin side chains and the OH groups of the carbohydrate (Gabius *et al*., 2011).

Cross adsorption studies would enable us to know whether the agglutinating activity of the sample is due to the presence of a single or more than one agglutinin (Millar and Ratcliffe, 1987; Mercy and Ravindranath, 1992). The results obtained in the present study shows that, all erythrocyte types tested, adsorbed the entire agglutinating activity from the sample leaving no residual volume to recognize the same or other erythrocytes used for the subsequent adsorptions revealing the presence of a single hemagglutinin as reported in *Cancer antennarius* (Ravindranath *et al*., 1985), *Scylla serrata* (Mercy and Ravindranath, 1992, 1993) and *Paratelphusa jacquemontii* (Maghil *et al*., 2003).

In general, the inhibitory ability of sialo glycoprotein reflects more the content of sialic acid rather than α2-3 and/or α2-6 linkage of the terminal sialic acid as well as N-and/or O-type glycosylation (Spiro and Bhayroo, 1974; Osawa and Tsuji, 1987). In the present investigation, fetuin and α acid glycoprotein are identified as the potent inhibitors of the agglutinin. The agglutinability is also inhibited by BSM, porcine thyroglobulin and lactoferrin. The nature of sialic acid in these glycoprotein inhibitors is N-glycolylneuranimic acid (NeuGc) (Mercy and Ravindranath,1993; Van Leeuwen*et al*., 2012). Presence of NeuGc in rabbit (Bhavananthan *et al*., 1964; Murayama *et al*.,1981), dog (Hashimoto *et al*., 1984), pig (Sarris and Palade, 1979) and ox (Kamerling *et al*., 1982; Rogers *et al*., 1986; Chien *et al*., 1978; Murayama *et al*.,1982) erythrocytes, agglutinated by the mucus agglutinin of the slug *L. alte* leads to the assumption that the mucus agglutinin of the slug *L. alte* may be NeuGc specific. The ability of the mucus agglutinin to agglutinate rabbit erythrocytes with high HA titer and its reduced ability to recognize asialo rabbit erythrocytes, the ability of the glycoproteins fetuin, α acid glycoprotein, bovine submaxillarymucin and lactoferrin to inhibit HA titer with great potency and their diminished potential following desialylation further supports the contention that the mucus agglutinin of the slug *L. alte* is sialic acid specific. The presence of sialic acid binding agglutinins in the gastropodanmollusk *L. alte*, incapable of synthesizing sialic acids and their occurrence in the pathogenic microbes suggest that this agglutinin may be involved in the innate immunity

of this organism. However, the exact specificity of the agglutinin can be ascertained only after affinity purification.

CONCLUSIONS

- Agglutinin with specific affinity for rabbit erythrocytes is identified in the mucus of the slug *L. alte*.
- Maximum agglutinability was observed at pH7.5, temperature 55^{o}C and in the presence of 1mM Ca^{2+}.
- Fetuin and α-acid glycoprotein are identified as the potent inhibitors.
- Results with a sialo erythrocytes and desialylated glycoproteins reveal the sialic acid specificity of the agglutinin.

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